2

The Principles of Freeze-Drying

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Summary

This chapter provides an up-to-date overview of freeze-drying (lyophilization) with particulars relevance to stabilizing live cells or viruses for industrial applications as vaccines or seed culture. The chapter discusses the importance of formulation, cycle development, validation, and the need to satisfy pharmaceutical regulatory requirements necessary for the commercial exploitation of freeze-dried products.

Key Words: Freeze-drying; lyophilization; lyoprotectants; secondary drying; sublimation.

1. Introduction

Water is essential to life, providing a universal solvent supporting biochemical activities within cells, which enables metabolism to continue and sustains all living processes. Quite simply, in the absence of water, life as we define it will cease, resulting in a state of death or dormancy in live cells or inhibiting biochemical activity in cellular extracts. Water also plays a major role in the degradation of stored material, providing conditions that potentiate autolysis, or promote the growth of spoilage organisms ***(1)***.

In order to stabilize labile products, it is therefore necessary to immobilize or reduce the water content of stored samples.

Vaccines, other biological materials, and microorganisms can be stabilized by chilling or freezing. However, maintaining and transporting samples in the frozen state is costly, whereas freezer breakdown may result in the complete loss of valuable product ***(2)***.

Alternatively, bioproducts can be dried in air using high processing temperatures. Traditional drying typically results in marked changes in the physical and chemical properties of the product by high solute concentration or thermal

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inactivation, and is more appropriate for dehydrating low-cost products such as foodstuffs.

Freeze-drying combines the benefits of both freezing and drying to provide a dry, active, shelf-stable, and readily soluble product ***(3,4)***.

1.1. Defining Freeze-Drying

Freeze-drying or lyophilisation describe precisely the same process. The term "lyophilization," which means "to make solvent loving," is less descriptive than the alternative definition "freeze-drying." Several alternative definitions have been used to describe freeze-drying. Operationally we could define freeze-drying as a controllable method of dehydrating labile products by vacuum desiccation.

Earlier accounts of freeze-drying suggested that ice was only removed by sublimation and defined this step as primary drying. The cycle was then described as being extended by secondary drying or desorption. Although these definitions are applicable to ideal systems, they incompletely define the process for typical systems that form an amorphous matrix or glass when cooled ***(5)***.

Technically, freeze-drying may be defined as:

1. Cooling of the liquid sample, followed by the conversion of freezable solution water into ice; crystallization of crystallizable solutes and the formation of an amorphous matrix comprising noncrystallizing solutes associated with unfrozen moisture.
2. Sublimation of ice under vacuum.
3. "Evaporation" of water from the amorphous matrix.
4. Desorption of chemiabsorbed moisture resident in the apparently dried cake.

1.2. History

The method can be traced back to prehistoric times and was used by the Aztecs and Eskimo for preserving foodstuffs. Toward the end of the 1880s the process was used on a laboratory scale and the basic principles understood at that time. Practically, the method remained a laboratory technique until the 1930s when there was the need to process heat-labile antibiotics and blood products. At this time, refrigeration and vacuum technologies had advanced sufficiently to enable production freeze-dryers to be developed, and since then the process has been used industrially in both the food and pharmaceutical industries ***(3,6)***.

Freeze-drying has a number of advantages over alternative stabilizing methods. These may be summarized by the following criteria ***(6)***:

1. The need to stabilize materials for storage or distribution.
2. The product may demand to be freeze-dried and there may be no suitable alternative available.

3. There may be a legal requirement to freeze-dry the product to satisfy regulatory demands.
4. Freezing will reduce thermal inactivation of the product and immobilize solution components.
5. Concentration effects such as "salting out" of proteins, alterations in the distribution of components within the drying and dried product, and so on, may be minimized by freeze-drying.
6. The water content of the dried product can be reduced to low levels, and in general samples are more shelf-stable when dried to low moisture contents, although overdrying may reduce shelf stability in sensitive biomaterials.
7. Because the product is normally sealed under vacuum or an inert gas, oxidative denaturation is reduced.
8. Loss of water equates to a loss of product weight and this may be important where transport costs are significant.
9. Sample solubility, shrinkage, unacceptable appearance, or loss of activity may all be improved when freeze-drying is used rather than an alternative technique.
10. Dispensing accuracy may be facilitated when the sample is dispensed as a liquid rather than a powder.
11. Particulate contamination is often reduced when samples are freeze-dried rather than spray or air-dried.
12. The need to compete with competitors supplying similar products.
13. The requirement to launch a product on the market while less costly drying techniques are being developed.
14. The production of intermediate bulk or requirement to remove solvents such as ethanol.
15. The need to maximize investment in drying plants by freeze-drying a minor product rather than invest in an alternative and costly drying process.
16. The need to separately dry two or more components that would be incompatible if dispensed together within a single container.

1.3. Types of Freeze-Dried Products

Freeze-dried products may be classified as:

1. Nonbiologicals, where the process is used to dehydrate or concentrate reactive or heat-sensitive chemicals.
2. Nonliving bioproducts. These comprise the major area of application and include enzymes, hormones, antibiotics, vitamins, blood products, antibiotics, inactivated or attenuated vaccines, and so on. This subgroup includes pharmaceuticals, which may be used diagnostically or therapeutically.
3. Bone and other body tissues for surgical or medical use; foods where organoleptic properties are important; industrial bioproducts.
4. Living organisms for vaccine or seed culture use, which must grow and multiply to produce new progeny after drying and reconstitution.
5. Miscellaneous, for example flood-damaged books, museum artifacts, and so on.

However, freeze-drying is less appropriate for:

1. Oily or sugar-rich materials where the medium does not freeze.
2. Products that form impervious surface skins, thereby preventing vapor migration from the drying sample during processing.
3. Eukaryote cells, which are able to retain viability when frozen only in the presence of additives, may be incompatible with the freeze-drying process.

2. The Process of Freeze-Drying

2.1. Description of Process

For convenience, the freeze-drying process may be divided into a number of discrete steps that may be summarized as ***(7,8)***:

1. For the processing of cell or other bioproducts a variety of preparatory processing steps may be required, e.g., vaccine preparation, extraction, purification, and formulation in a suitable medium for freeze-drying.
2. Sample freezing, which reduces thermal denaturation of product, immobilizes solution components, and prevents foaming when the vacuum is applied. Freezing also induces a desired ice-crystal structure within the sample, which facilitates drying.
3. Primary drying (sublimation) where conditions must be maintained in the drying chamber to sustain water migration from the sample ice during drying. During primary drying the sample temperature (strictly freeze-drying interface temperature) must be maintained below the eutectic, glass transition, collapse, or melt temperature as appropriate to minimize sample damage during drying.
4. A secondary drying stage during which resident moisture adsorbed to the apparently dry structure is removed by desorption.
5. Sealing the dried sample in a vacuum or under an inert gas at the end of the process, both of which exclude the entry of reactive, destabilizing, atmospheric gases such as oxygen or carbon dioxide into the dried sample and prevent the ingress of damp air into the freeze-dried sample. (Note that a freeze-dried product will have a vastly expanded dry surface area and is therefore particularly sensitive to air denaturation or moisture readsorption.)
6. The samples are then removed from the freeze-dryer, stored and/or distributed for use prior to reconstitution for injection, application, or regrowth.

2.2. Processing Principles

Freeze-drying is a complex process during which drying may proceed more or less rapidly within individual samples throughout the process batch, such that parts of the product will be frozen, whereas other areas are drying or will have dried depending on the nature of the sample and stage in the cycle. The precise freezing and drying behavior will be determined by the interrelationship between the sample and shelf temperature, system pressure, extent of product dryness, and variations in drying conditions throughout the cycle.

Often regarded as a gentle method of drying materials, freeze-drying is in reality a potentially damaging process where the individual process stages should be regarded as a series of interrelated stresses, each of which can damage sensitive bioproducts. Damage sustained during one step in the process may be exacerbated at succeeding stages in the process chain and even apparently trivial changes in the process, such as a change in container, may be sufficient to transform a successful process to one which is unacceptable ***(8)***.

Freeze-drying will not reverse damage incurred prior to formulation and care must be exercised when selecting an appropriate cell type or technique used to culture or purify the cell or its extracts prior to freeze-drying. The essence of the formulation exercise should be to minimize freeze-drying damage, loss of viability, or activity. To ensure minimal losses of activity, the sample may require dilution in a medium containing protective additives, specifically selected for the product or application. Although frequently described as "protectants" these additives may not be effective at all stages of the process but may protect only during particular steps in the drying cycle. At other stages, the additive may fail to protect the active component and indeed may be incompatible with the process. It is also important to appreciate that individual stages in the process can result in damage, which initially remains undetected, becoming evident only when the dried sample is rehydrated. Particular attention must be applied to the selection and blending of the additive mixes in the formulation and the importance of formulation will be discussed at greater length later ***(1,9–12)***.

Freeze-dried products should be:

1. Minimally changed by the process.
2. Dry.
3. Active.
4. Shelf stable.
5. Clean and sterile (for pharmaceutical applications).
6. Ethically acceptable.
7. Pharmaceutically elegant.
8. Readily soluble and simple to reconstitute.
9. Process should be economically practicable.

Products should be formulated to ensure batch product uniformity, whereas there may be particular requirements relating to product use. In this context, vaccines freeze-dried for oral or aerosol delivery may require the inclusion of excipients that minimize damage when the dried product is exposed to moist air ***(11)***.

A wide range of containers can be used to freeze-dry vaccines, microorganisms, and others, including all glass ampoules, rubber stoppered vials, double

chambered vials, and prefilled syringes that hold both dried vaccine and diluent, bifurcated needles, and so on. Alternatively, vaccines can be dried in bulk in stainless steel or plastic trays and the resultant powder tableted, capsulated, sachet filled, or dispensed into aerosol devices for lung or nasal delivery.

2.3. Sample Freezing

Regarded as the first step in the process, the formulated product must be frozen before evacuating the chamber to induce sublimation ***(13,14)***. Freezing will:

1. Immobilize the components in the solution and prevent foaming as the vacuum is applied.
2. Reduce thermal inactivation of the dispensed product.
3. Induce a specific ice-crystal structure within the frozen mass, which will facilitate or inhibit vapor migration from the drying cake. In short, the ice structure formed during freezing will dictate subsequent freeze-drying behavior and the ultimate morphology of the dried cake.

Ideally freezing should minimize solute concentration effects and result in a sample where all the components are spatially arranged as in the dispensed solution. However, it may not be possible to achieve this ideal when typical solutions or suspensions are frozen. When addressing the freezing of aqueous solutions or suspensions, there is the need to consider both the solvent (water in the case of aqueous solutions) and solute(s) in the formulation.

Frequently, the terms cooling and freezing are erroneously interchanged and confusion in understanding the process may occur and may be compounded by failing to distinguish between shelf or product cooling and freezing. Cooling refers to the reduction of temperature of the freeze-dryer shelves, the diatherm fluid circulating through the shelves, the vial and tray mass, interior of the freeze-dryer, and the dispensed solution or suspension. Cooling does not assume a change in state from liquid to solid and strictly should be used to describe reducing temperature during the initial stage of freeze-drying. Freezing refers to the abrupt phase change when water freezes as ice. Except for very complex biomolecules or cold sensitive cells, cooling in the absence of freezing (chilling) is generally not damaging to biomaterials.

When solutions or suspensions are frozen, they may cool appreciably below their measured freezing point prior to ice formation, a phenomenon defined as supercooling (undercooling or subcooling). The extent of supercooling depends on cooling rate, sample composition and cleanliness, dispensed fill volume, container type, method of sample cooling, and so on. Even when a simple solution is repeatedly cooled or warmed, the onset and extent of supercooling will vary from cycle to cycle. In the supercooled state, while the composition of the solution remains unchanged, the cooled liquid is thermodynamically unstable and sensitive to ice

formation. As the solution is cooled to lower temperatures, the probability of ice crystallization will correspondingly increase. For optimized freeze-drying, the intention should be to induce supercooling in the suspension to encourage uniform cooling and freezing throughout the sample contents ***(15–17)***.

Sample freezing may be defined as the abrupt conversion of the suspension into a mixture of ice and solute concentrate. Freezing is a two-step process during which water initially nucleates, followed by the growth of the ice crystals that pervade the solute phase resulting in a mixture of ice and solute concentrate. Under typical processing conditions, ice nucleates heterogeneously around microscopic particles within the suspension and is encouraged by reducing temperature and agitating the supercooled suspension to increase the probability of contact between nucleating foci and water clusters. Nucleation depends on the number and physical nature of particulate impurities within the suspension or solution. Ice is a particularly effective nucleation focus and cryobiologists may deliberately seed samples with ice to induce nucleation. Other effective ice nucleators include glass shards and specifically formulated nucleation promoters. Whereas nucleation aids can be added to experimental systems, deliberate attempts to add ice inducers to pharmaceutical materials would be at variance with Good Pharmaceutical Manufacturing Practice ***(18)***.

In contrast to nucleation, ice growth (proliferation) is encouraged by raising the temperature, thereby decreasing the suspension viscosity. Ice nucleation and proliferation are inhibited at temperatures below the glass transition temperature (Tg′), whereas above the melting temperature (Tm) the suspension or solution will melt. The consequences and measurements of these parameters are important elements in the formulation exercise ***(14,18,20,21)***.

To facilitate the sublimation of water vapor from the drying mass, the ice crystals should be large, wide, and contiguous, extending from the product base toward its surface, thereby providing an optimized structure for vapor migration. Crystal structures commonly observed during freeze-drying when solutions are frozen in trays or vials include dendritic structuring, where the ice crystal branches continuously from the nucleating focus and the spherulite form, and where subbranching is discouraged because the solution viscosity is high, or fast rates of cooling are used.

Cooling or freezing rates are defined as slow (suboptimal), rapid (superoptimal), or optimal as assessed by criteria such as postfreezing cell survival or biopolymer activity, and is ambiguous unless conditions are more precisely defined. Cooling rates may be defined in terms of:

1. The rate at which the shelf temperature is cooled per unit time.
2. The rate at which the solution cools per unit time.
3. The depth of liquid within the vial (in mm) which cools per unit time.

2.3.1. Shelf-Cooling Rate

The shelf-cooling rate ***(22)*** is the simplest parameter to control and programmed rates of cooling are standard options on research and production freeze-dryers. Because shelf temperature and product responses are not identical, defining shelf-cooling rate will not fully define product behavior. Although we are concerned with the cooling rate achievable within each vial, this parameter is less easy to monitor compared with shelf cooling, and freeze-drying cycles generally are controlled by programmed shelf cooling rather than feedback control from the sample. Cooling rates of the product/cell suspension will vary considerably from vial and throughout the sample within the vial and, consequently, measuring the temperature of vial contents at a fixed position will give only an approximation of the sample temperature variation.

Observing the freezing pattern of a number of vials arranged on a shelf will demonstrate that while the contents of some vials will freeze slowly from the vial base, neighboring vials may remain unfrozen and supercool appreciably before freezing instantly. This random freezing pattern will reflect differences in ice structure from vial to vial and translated into different drying geometries from sample-to-sample vials. In summary, freezing patterns will be related to:

1. The ice forming potential within each vial.
2. The relative position of the vial on the shelf causing exposure of individual vials to cold or hot spots.
3. Edge effects where samples in vials on the periphery of each shelf will be subjected to heat transmitted through the chamber walls or door.
4. The insertion of temperature into the sample, which will induce ice crystallization.
5. The evolution of latent heat as samples freeze, which will tend to warm adjacent containers.
6. Variations in container base geometry, which may impede thermal contact between sample and shelf.

The ice and solute crystal structure resulting from sample freeze has a major impact on subsequent freeze-drying behavior, encouraging the sample to dry efficiently or with defects such as melt or collapse depending on freezing rate used. The preferred ice structure comprising large contiguous ice crystals is induced by freezing the sample at a slow rate of c. 0.2–1.0°C/min. Slow cooling will also induce the crystallization of solutes reluctant to crystallize when faster rates of cooling are used. However, a slow rate of cooling may exacerbate the development of a surface skin, which inhibits sublimation efficiency (*see* **Subheading 3.6.2.**). Slow cooling can also inactivate a bioproduct by prolonging sample exposure to the solute concentrate biomolecules. However, a fast rate of cooling can result in the formation of numerous, small, randomly

orientated ice crystals embedded in an amorphous solute matrix, which may be difficult to freeze-dry. Complicating the choice of freezing regimes is the fact that the optimal cooling rate cannot be sustained where the sample fill depth exceeds 10 mm. In short, defining cooling rates often requires a compromise in sample requirements.

2.3.2. Ice Structure and Freeze Consolidation **(13)**

A period of consolidation (defined as the hold time), is necessary at the end of sample cooling to ensure that all the vial contents in the sample batch have frozen adequately, although excessive hold times will increase the time of sample freeze and impact on the overall cycle time. It is a fallacy to assume that the ice structure induced remains unchanged during this consolidation period and an ice structure comprising a large number of small ice crystals, induced by rapid cooling, is thermodynamically less stable than an ice structure comprising fewer, larger crystals. The thermodynamic equilibrium can be maintained by recrystallization of ice from small-to-large crystals, a process termed grain growth. Although ice structure changes take place randomly from vial to vial, the hold period is a major factor in ice recrystallization resulting in significant variation in crystal structure and subsequent sublimation efficiency from sample to sample the longer the hold period is employed.

As an alternative to increasing the length of the hold time to encourage ice recrystallization, a more controlled and time-efficient method of inducing recrystallization is to heat anneal the frozen sample ***(23)***. Essentially heat annealing is achieved by:

1. Cooling the product-to-freeze solution water and crystallized solutes.
2. Raising the product temperature during the freezing stage to recrystallize ice from a small to a large ice crystal matrix. (**Note**: this warming phase may also crystallize solutes that are reluctant to crystallize by cooling [*see* **Subheading 2.3.3.**].)
3. Cooling the product to terminal hold temperature prior to chamber evacuation.

Heat annealing (also defined as tempering) is particularly useful to:

1. Convert an ice structure to a crystalline form, which improves sublimation efficiency.
2. Crystallizing solutes that are reluctant to crystallize during cooling.
3. Provide a more uniform, dry structure throughout the product batch.
4. Integrated with rapid cooling, heat annealing may minimize the development of a surface skin on the sample thereby facilitating sublimation.
5. Because heat annealing induces a more porous cake structure with improved drying efficiency, a lower dried sample moisture content may be achieved, with improved solubility.

Although heat annealing will increase the length of the freezing stage of the cycle, overall freeze-drying cycle times may be significantly reduced because of improvements in drying efficiency resulting from heat annealing (*see* **Fig. 1**).

Care should be exercised when selecting temperatures and hold times for heat annealing, particularly when defining the upper temperature for sample warming (*see* **Subheading 2.3.3**). Subjecting a labile product, such as a vaccine, to temperatures above the eutectic temperature will expose the sample to hypertonic solution concentrates as the sample partially melts, which can damage sensitive biomolecules.

2.3.3. Freezing Solute Behavior **(6,24–27)**

Regardless of the precise freezing pattern, the formation of ice will concentrate the remaining solution within the container. As the proportion of ice increases within the mixture, solute concentration will correspondingly increase. In the case of an aqueous 1% (w/v) saline solution, this concentration effect will be considerable, increasing to approx 30% (w/v) just prior to freezing, and damage to biomolecules results as a consequence of solute concentration exposure rather than direct damage by ice crystals. The behavior of the solute(s) within the solute concentrate depends on the nature, concentration, cooling rate, and interactions between individual solutes present in the medium and forms the basis for experimental review during a formulation development exercise.

Overall, four patterns of solute response are observed during freeze-drying:

1. Solute crystallizes readily, regardless of cooling rate or freezing conditions, to form a mixture of ice and solute crystals (this behavior is termed eutectic freezing).
2. Solute crystallizes, but only when the solution is subjected to a slow rate of cooling.
3. Solute crystallizes only after the solution has been heat annealed.
4. Solute fails to crystallize regardless of cooling rate or regime adopted, and solute remains associated with unfrozen water as a metastable amorphous mass or glass.

For a crystallizing solute, the eutectic point is the lowest temperature in a system in which a residual liquid phase and solid phase are in equilibrium. Above the eutectic point, ice and solute concentrate persist, whereas below the eutectic point, a mixture of ice and solute crystals is formed. Eutectic temperatures for aqueous solutions containing crystallizing salts are characteristic for each solute and are significantly below the freezing point of water (for example, eutectic temperature for sodium chloride = –21.4°C). Exposing cells or proteins for prolonged periods to a eutectic solution comprising hypertonic salt concentrations can cause damage by plasmolysis or precipitation by "salting out" ***(28)***.

The eutectic zone is the range of temperatures encompassing all the eutectic temperatures within the system. For a two-part water/solute system, the eutectic temperature is a discrete, quantifiable temperature in contrast to multisolute systems where a eutectic zone may be observed that represents a range of

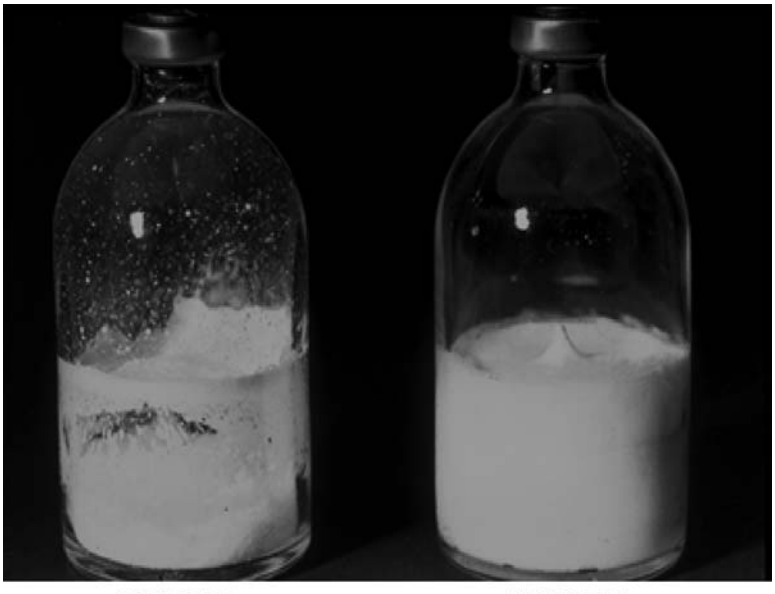

Fig. 1. Samples of freeze-dried products freeze-dried using an unsuitable process cycle (sample A) compared with an identically formulated sample (sample B) where processing conditions have been optimized to provide an acceptable product. Both samples are formulated in an identical medium and filled to a depth of 60 mm, which greatly exceeds the recommended fill depth maximum for freeze-drying of 10 mm. Although such excessive fill depths are often required for commercial or marketing reasons, such fills greatly impede sublimation resulting in prolonged cycles, collapsed product, poor sample solubility, reduced product activity and shelf stability. In addition, such samples often exhibit high-moisture content, unacceptable pharmaceutical elegance, and display cake fracture and physical loss of sample from the vial as drying progresses (defined as ablation and of particular concern when live vaccines or cytotoxic drugs are freeze-dried). Sample A was freeze-dried using a conventional freezing and drying cycle and resulted in a cycle time in excess of 10 d with extensive vial breakage, in addition to the unacceptable features noted previously. Sample B was dried using a cycle designed to ensure satisfactory freezing to induce an optimized frozen structure conducive to rapid rates of sublimation. In addition, the author was able to accelerate drying by adjusting shelf temperature and chamber pressure so that drying times were reduced to 3 d. Product quality was assured by maintaining sample temperatures sufficiently below collapse (Tc) or glass transition (Tg′) temperature, defined during formulation and process development.

temperatures where the minimum eutectic temperature is lower than that of any individual eutectic temperatures in the medium.

Typical freeze-dried vaccine formulations fail to crystallize completely when cooled, and a proportion of the solutes in the sample persist as an amorphous,

noncrystalline, glass. When exposed to temperatures above their glass transition (Tg′) or collapse temperature (Tc), these samples may warm during sublimation causing the amorphous mass to soften so that freeze-drying progresses with sample collapse to form a sticky, structure-less residue within the vial. Less severe collapse will result in the formation of a shrunken, distorted, or split cake ***(20,29–33)*** (*see* **Fig. 1**).

Collapsed cakes are not only cosmetically unacceptable but may be poorly soluble, exhibit reduced activity, or compromised shelf stability. Collapse may be exacerbated by the formation of a surface skin, which impedes vapor migration from the drying structure. To avoid sample collapse, it is necessary to maintain the sublimation interface below Tg′ or Tc throughout primary drying and to include excipients in the formulation, which reduce the severity of collapse. It is therefore essential to characterize formulations experimentally during the process development program. Although collapse may cause operational difficulties during freeze-drying, the induction and maintenance of the amorphous state may be essential for protecting labile biomolecules during freezing, drying, and storage ***(23,34,35)***.

2.3.4. Freezing in Practice

Samples may be frozen in a variety of ways depending on operational requirements:

1. Samples may be frozen in a freezer or a cooling tunnel prior to transfer to the freeze-dryer for desiccation. Advantages include increased annual sample throughput because the freeze-dryer is used only for drying. Disadvantages include the greater risk of sample melt or contamination resulting from the need to transfer samples from the freezer into the drier.
2. Samples may be frozen in the absence of cooling by evacuating the container and relying on evaporative cooling to freeze the sample. However, the need to prevent sample foaming when the dryer is evacuated precludes the widespread use of the method.
3. Pellet freezing. Strictly this is not a method of freezing but can be useful when bulk products, including vaccines for subsequent powder filling, are processed. The suspension is sprayed into a cryogenic liquid or onto a cold surface to form frozen sample droplets, which are then be placed into trays or flasks for freeze-drying. Under these conditions, sublimation rates are typically very high because the thickness of the dry layer is restricted only by the pellet radius, and drying proceeds in a virtually unimpeded manner from each pellet.
4. The most widely used technique is to freeze the samples directly on the freeze-dryer shelf. Although this method has the disadvantage that the drier is used for part of the cycle as freezer, freezing and drying samples within a single machine eliminates the need to transfer samples from freezer to drier, and therefore improves sample cleanliness while reducing product vulnerability.

3. The Process of Water Removal

3.1. Sublimation and Drying (34)

Under atmospheric conditions, liquid water is converted into vapor by warming, a process defined as evaporation. However the three states of water ice, liquid, and vapor coexist at the triple point and illustrates that at subatmospheric pressures ice can convert directly to vapor by sublimation. Ice sublimation from a frozen sample results in an open, porous, dry structure where solutes are spatially arranged as in the original solution or suspension. In contrast to evaporation, where components are concentrated as drying progresses, sublimation under vacuum minimizes concentration effects providing a dry product that is active and readily soluble.

Having frozen the solution, the next step is to dry the sample by subliming ice directly into water vapor. In order to maintain freeze-drying conditions, it is essential to lower the partial pressure of water below the triple point (approx 800 mBar at 0°C) , to ensure the direct conversion of ice into water vapor and prevent sample melt. Vacuum will (1) reduce the air concentration above the product and encourage sublimation, and (2) ensure that air leaking into the system is removed.

3.2. Sublimation Rate and Chamber Pressure Conditions

Decreasing the chamber pressure below 0.8 mBar will increase the rate of sublimation by reducing the gas/vapor concentration above the sample to provide minimal resistance to water molecules migrating from the sample. Sublimation rate continues to increase, reaching a maximum at approximately mBar. Reducing system pressure further will not increase sublimation rate and indeed, contrary to expectations, at very low system pressures the sublimation rate will decrease.

This apparent paradox can be explained by assuming that two separate factors influence sublimation efficiency:

1. System pressure reduction sufficient to “thin” the chamber atmosphere and facilitate vapor migration from the sample.
2. A system pressure containing sufficient gas or vapor molecules in the chamber to conduct heat energy from the shelf into the sample. Essentially, under high-vacuum conditions a thermos flask effect is induced in the chamber, which inhibits heat transfer from the shelf. Under high-pressure (poor vacuum) conditions, heat transfer from the shelf to the sample is gas/vapor conduction in contrast to high-vacuum conditions where heat transfer by conduction is reduced and product heat is predominantly by radiation, which is a relatively inefficient mechanism.

Industrial and development freeze-dryers are routinely operated at a constant chamber pressure to facilitate heat transfer into the sample, and system pressure can be controlled by (1) bleeding air into the chamber, condenser, or vacuum

system, or (2) isolating the pump from the chamber to raise chamber pressure by increasing the number of water molecules migrating from the sample into the chamber.

Either method is equally effective. It is important to appreciate that improving the drying rate by bleeding air into the chamber does not "blow" water molecules from the product ***(36)***.

3.3. Vapor Differential Pressure and Drying Efficiency (36,37)

To sustain freeze-drying it is necessary to establish a pressure gradient from a sample (highest pressure), to condenser, and finally vacuum pump (lowest pressure) so that water migrates from the sample as drying progresses. Although the temperature of the sample must be higher than that of the condenser to ensure a net migration of water from the sample, the system driving force represents the difference in vapor pressure (VP) rather than the difference in temperature between sample and condenser, and can be calculated as the difference in VP between the two. For example, sample at –20°C has a VP = 0.78 torr and with the condenser at –40°C (equivalent to a VP of 0.097 torr), driving force will be 0.78–0.097 or 0.683 torr. Little improvement in driving force is achieved by operating the condenser at –70°C. (VP = 0.002 torr, providing a VP differential of 0.78 [sample] – 0.002 [condenser] of 0.778 torr.)

The example illustrates that greater sublimation efficiency is derived by increasing sample temperature rather than reducing condenser temperature, and the selection of suitable excipients that enable high processing temperatures to be used during freeze-drying without compromising sample quality plays an important role in process and cycle development.

3.4. Heat and Mass Transfer (36,37)

The essence of the freeze-drying process depends on maintaining a critical balance between the conversion of ice into water vapor by sublimation under vacuum and the removal of that vapor from the frozen mass. To maintain sublimation, heat energy is applied to the product to compensate for sublimation cooling. However, the heat extracted from the drying sample as water vapor must carefully balance the amount of energy added to the sample. Unless this equilibrium can be maintained, the product temperature will either decrease thereby reducing drying efficiency or increase, which may compromise product quality by inducing melt or collapse. This critical balance between sample warming to increase drying rate and vapor extraction is defined by the heat and mass transfer equation. In the early stages of sublimation the equilibrium is simple to maintain because the dry structure offers minimal resistance to vapor flow. However, as drying progresses and the depth of the dry layer increases, impedance to vapor flow will also increase and the sample

may warm sufficiently to melt or collapse unless the process temperature is reduced. One consequence of reducing the energy input will be to reduce drying rate and prolong cycle times, but this may be unavoidable if sample quality is to be preserved.

3.5. Cooling and Warming the Product (7,14,22)

The shelves fitted into the freeze-dryer to support sample containers may be alternatively cooled to initially freeze the sample or maintain shelf at a constant temperature throughout the drying cycle or warmed to provide energy for drying. Basically two systems may be fitted:

1. An independent cooling coil is embedded in the shelf through which cold refrigerant is supplied (this system is termed direct expansion) and a heating element is bonded into or onto the base of the shelf. Shelf control is maintained by alternately operating either the heater or cooler. Direct expansion systems are relatively inexpensive but fail to achieve temperature control much better than ±5°C.
2. For industrial or development activities, where shelf control to ±1°C is necessary to meet good manufacturing practice (GMP) requirements, a diatherm fluid, which is invariably silicone fluid, is circulated through the shelves and a separate refrigerator/heat exchanger maintains the diatherm fluid at a preset temperature.

The mechanism and the relative quantities of heat entering the product will depend on:

1. The nature of the product, its fill depth, consistency, and so on.
2. The dimensions and geometry of the sample container and whether the container rests directly on a shelf or is supported in a tray.
3. The freeze-dryer design.
4. Chamber vacuum conditions.

Product temperature can be maintained by either raising or reducing shelf temperature or by alternating system pressure which has the effect of improving; or reducing heat transfer efficiency as outlined in **Subheading 3.1.** Regardless of the precise system incorporated into the freeze-dryer, shelf temperature conditions may be controlled manually or programmed using a PC or microprocessor control.

3.6. The Drying Cycle

For clarity it is usual to separate the drying cycle into primary drying (the sublimation stage) and secondary drying or desorption.

3.6.1. Primary Drying

The first step in the drying cycle is defined as primary drying and represents the stage where ice, which constitutes between 70 and 90% of the sample

moisture, is converted into water vapor. Sublimation is a relatively efficient process although the precise length of primary drying will vary depending on the sample formulation, cake depth, and so on. During primary drying, the sample dries as a discrete boundary (the sublimation interface), which recedes through the sample from surface to base as drying progresses.

3.6.2. The Sublimation Interface **(7,8,14,32,33)**

Variously described as the drying front, freeze-drying front, and so on, macroscopically the sublimation interface can be observed as a discrete boundary that moves through the frozen sample to form an increasingly deeper layer of dried sample above the frozen sample. Heat is conducted from the shelf through the vial base and the frozen sample layer to the sublimation front where ice is converted into water vapor. Several consequences result from this progressive recession of the sublimation front through the dry layer, which include:

1. The maintenance of the frozen zone at a low temperature because of sublimation cooling.
2. An increase in the resistance to vapor migration and a decrease in sublimation rate as the dry layer increases in thickness.
3. Because the sublimation interface represents a zone representing maximum change of sample temperature and moisture content, the interface represents the zone over which structural softening or collapse is likely to occur.
4. Water migrating from the sublimation front can reabsorb into the dried material above the sublimation interface.

Because the sublimation interface is the region where freeze-drying takes place, temperature monitoring of the interface is of paramount importance for product monitoring. However, because the sublimation front is constantly moving through the sample, interface temperature cannot be effectively monitored using traditional temperature probes. Although the sublimation interface is defined as a discrete boundary, this is true only for ideal eutectic formulations, where ice crystals are large, open, and contiguous with each other. For typical amorphous formulations, such as vaccines, the sublimation front is much broader and comprises individual ice crystals imbedded in the amorphous phase. Under these conditions, although ice sublimes within the isolated crystals, the water vapor must diffuse through the amorphous phase (which is itself progressively drying) until it can migrate freely from the drying sample matrix. Under these conditions, sublimation rates are much lower than those anticipated from data derived using eutectic model systems. Complicating a precise prediction of sublimation rate is the fact that fractures in the dry cake between the ice crystals can improve drying efficiency. All of these factors, including system impedances caused by the development of a surface skin on the sample, have to

be considered during sample formulation and cycle development programs. Notwithstanding these complications in precisely defining primary drying, sublimation is nevertheless a relatively efficient process and conditions used for primary drying include the use of shelf temperatures high enough to accelerate sublimation without comprising sample quality by inducing collapse or melt, combined with high system pressures designed to optimize heat conduction from shelf into product. Removing the product when sublimation has been judged as complete will provide a vaccine which appears dry but which displays a high-moisture content that is invariably too high (7–10%) to provide long-term storage stability, and the drying cycle is extended to remove additional moisture by desorption or secondary drying.

3.6.3. Secondary Drying

In contrast to primary drying, which is a dynamic process associated with high vapor flow rates, secondary drying is much less efficient with secondary drying times representing 30–40% of the total process time but only removing 5–10% of the total sample moisture. Under secondary drying conditions, the sample approaches steady-state conditions where moisture is desorbed or absorbed from or into the sample in response to relative humidity and shelf temperatures. Desorption is favored by increasing shelf temperature, using high-vacuum conditions in the chamber, thereby reducing the system vapor pressure or relative humidity. Conversely, when the shelf temperature is reduced and the vapor pressure in the system increased by warming the condenser, dried samples will reabsorb moisture and exhibit an increase in moisture content. Although sample collapse during secondary drying is generally less likely than collapse during primary drying, it is possible to induce collapse in the dried matrix by exposing the sample to temperature above its glass transition temperature (Tg).

3.6.4. Stoppering the Product

A freeze-dried product is both hygroscopic and has an enormously exposed surface area. Consequently, exposing the dried product to atmosphere will result in reabsorption of damp air into the product. Both water and air are damaging to a dried sample, causing degradative changes resulting in poor stability and it is therefore prudent to stopper samples within the freeze-dryer prior to removal. Stoppering under a full vacuum provides ideal conditions for ensuring product stability because reactive atmospheric gases are reduced to a minimum. However, injecting water into a sample in a fully evacuated vial can induce foaming, which can be reduced by filling vials with an inert gas, such as nitrogen, before stoppering.

4. Reconstituting the Product

It is often supposed that because freeze-drying only removes water, then all products will be fully active by rehydrating only with water. This may not be the case and freeze-dried products often exhibit enhanced activity when reconstituted in an isotonic medium, such as saline, rather than water.

5. Freeze-Dryer Design *(6,22,38–40)*

The need to operate the freeze-dryer under low-pressure conditions to convert ice directly into water vapor (a process termed sublimation) adds to the complexity and cost of dryer because the chamber holding the sample must withstand the differential pressure from vacuum to atmosphere. Although a suitable vacuum pump is essential for initially evacuating the chamber and eliminating air that may leak into the dryer during operation, vacuum pumps are not capable of continuously removing water vapor subliming from the sample and a refrigerated trap (termed the process condenser) must be placed between the sample and the pump to condense the moisture migrating from the drying sample. In reality, it is the condenser that comprises the "pumping force" of the system. Process condensers may be incorporated into the drying chamber (referred as an internal condenser) or located in a separate chamber between the sample chamber and pump (external condenser). Each geometry has advantages and disadvantages although either design may be used. Stainless steel is typically used to fabricate research or production dryers because this metal can be cleaned by a wide range of sanitizers including steam. For GMP manufacture the freeze-dryer is invariably sterilized by pressurized steam and this adds to the complexity and expense of the dryer because it has to conform to the requirements to operate under subatmospheric and pressure conditions. Modern freeze-dryers are also fitted with internal stoppering devices for sealing vials at the end of the cycle, valves and monitoring devices for assessing drying efficiency, and are typically computer or microprocessor controlled so that cycles can be reproduced and evaluated for regulatory purposes. When freeze-drying vaccines, it may be necessary to incorporate protective devices and introduce processing protocols that ensure both safe operation and prevent product cross-contamination.

6. Sample Damage During Freeze-Drying *(13,23,27,31–33)*

Damage to a freeze-dried product may occur:

1. When the solution is cooled (described as cold or chill shock).
2. During freezing as the ice forms and the unfrozen solute phase concentrates.
3. During drying, particularly when the sample collapses as drying progresses.
4. By protein polymerization when high shelf temperature are used for secondary drying.

5. During drying and storage because of damage by reactive gases, such as oxygen, and it is important to appreciate that even in a vacuum, sufficient gas molecules will be present in the sealed sample to cause inactivation.
6. During storage by free radical damage or Maillard reactions.
7. During reconstitution, particularly if the sample is poorly soluble.

6.1. Chill Damage (Cold Shock)

Reducing temperature in the absence of ice formation is generally not damaging to biomolecules or live organisms, although sensitive biopolymers may be damaged by cold shock ***(39)***.

6.2. Freezing Damage (9,28)

Reducing temperature in the presence of ice formation is the first major stress imposed on a biomolecule. Direct damage by ice is not generally damaging except when living cells are frozen at very fast rates, which may induce the formation of intracellular ice within the cell. Biomolecules are more likely to be damaged by an increase in solute concentration as ice forms. We have described how bacteria can remain fully viable when cooled to –18°C in the absence of ice formation, but when frozen to this temperature, viability was reduced to 60%. Freezing will result in:

1. Ice formation ***(40)***.
2. A rise in solute concentration (this effect can be appreciable and a 1% solution of sodium chloride will increase to 30% by freeze concentration as ice forms) ***(41)***.
3. Changes in solution tonicity ***(42)***.
4. Concentration of all solutes, including cells and biomolecules that are encouraged to aggregate ***(43,44)***.
5. An increase in solute concentration that may result in "salting out" of protein molecules ***(43)***.
6. Differential crystallization of individual buffer salts resulting in marked changes in solution pH as the solution freezes ***(44)***.
7. Concentration of potentially toxic impurities above a toxic threshold sufficient for the impurities to become toxic ***(44)***.
8. Disruption of sulfur bonds.
9. Generation of anaerobic conditions as freezing progresses.

7. Factors Effecting Dried Products

Freeze-dried vaccines should be formulated to minimize storage decay and should tolerate storage at ambient temperatures for distribution purposes. However, it is a fallacy to suppose that a freeze-dried product remains immune to damage during storage and factors which damage freeze-dried products include:

1. Temperature. Whereas a freeze-dried product is more shelf stable than its solution counterpart, freeze-dried materials are sensitive to thermal decay and will be influenced by storage temperature ***(45)***.
2. Moisture content ***(46–53)***.
3. Reactive gases ***(54)***.
4. Light.
5. Free radical damage ***(55)***.
6. Background nuclear radiation.
7. Specific chemical reactions including Maillard reactions ***(56)***.

The interrelationship between sample formulation, dried cake moisture cake, storage conditions, and glass transition temperature (Tg) are complex. In general terms, any physical distortion of the dry cake during storage will often result in a much more rapid loss of sample activity than predicted using the Arrhenius equation for reviewing similar samples ***(21,29,32,33,57–59)***.

7.1. Influence of Suspending Medium Composition on Survival of Live Cells to Freeze-Drying

Attempts to freeze-dry cells in water or a simple salt solution typically result in poor survival. A wide range of protective media has been developed for preserving freeze-dried vaccines, including augmented growth media or sugar solutions. Carbohydrates are widely used as freeze-drying protectants either individually or in combination with other solutes. They should be chosen on the basis of experimentally determining their freeze-drying characteristics rather than on a pragmatic basis. Monosaccharides, such as glucose, provide good bioprotection during freezing and freeze-drying but exhibit low glass transition (Tg′) or collapse temperatures (Tc) and dry with collapse when orthodox freeze-drying cycles are used. Disaccharides are effective freeze-drying protectants, and because they display higher collapse than monosaccharides, freeze-dry successfully when conventional drying cycles are used. Reducing sugars may induce damaging Maillard reactions, thereby compromising stability, and for this reason nonreducing disaccharides, such as sucrose or trehalose, are preferred to reducing sugars such as lactose ***(62)***. The addition of salts to formulations containing sugars will markedly depress Tg′ or Tc. ***(1,3,20,23,31,58,60)***.

Although presenting technical difficulties such as sample collapse, during freeze-drying the amorphous phase may be an essential prerequisite for stabilizing biomaterials, such as vaccines and live cells, by providing an integration of the protective additive, and biomolecule thereby minimizing damage level during freeze-drying and drying.

It is not possible in a review of this length to provide anything other than an overview of freeze-drying relevant to the freeze-drying of labile biomolecules and both inactivated or attenuated vaccines. In producing this chapter, the

author nevertheless has attempted to examine all aspects of freeze-drying, because even a basic understanding of this complex technology requires an appreciation of all the factors in the process that can influence the acceptability or unacceptability, safety, and efficacy of the preparation.

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